Plant and Soil **210:** 103–113, 1999.

Isolation and characterization of diazotrophic bacteria from banana and pineapple plants

O.B. Weber[1,*], V.L.D. Baldani[2], K.R.S. Teixeira[2], G. Kirchhof[3], J.I. Baldani[2] and J. Dobereiner[2]

[1]*EMBRAPA – National Center for Tropical Agroindustry Research (CNPAT), P.O. Box 3761, Planalto Pici, 60511-110, Fortaleza, CE;*
[2]*EMBRAPA – National Center for Agrobiology Research (CNPAB), Km 47, Seropédica, 23851-970, RJ, Brazil;*
[3]*GSF – Institute of Soil Ecology, Neuherberg, D-85758 Oberschleissheim, Germany*

Received 2 December 1998. Accepted in revised form 27 April 1999

Key words: diazotrophic, plant-associated bacteria, *Musa* spp., *Ananas comosus*, microbial ecology

Abstract

Banana and pineapple fruit crops are widely cultivated in tropical areas where high amounts of fertilizers are applied, principally nitrogen. Over 200 kg $N.ha^{-1}.yr^{-1}$ is often applied to these crops. Nevertheless, developing countries face the problem of high costs of chemical fertilizers. As already demonstrated for other tropical crops, like sugar cane, the utilization of nitrogen-fixing bacteria may support the growth of these fruit plants. In this work, we demonstrate the association of nitrogen-fixing bacteria with banana and pineapple. Samples from roots, stems, leaves and fruits of different genotypes showed the occurrence of diazotrophic bacteria, when evaluated in semi-specific semi-solid media. These isolates could be separated into seven different groups according to their morphological and physiological characteristics. Additional, phylogenetic assignments were performed with group- and species-specific oligonucleotide probes. Bacteria related to the groups of *Azospirillum amazonense*, *Azospirillum lipoferum*, *Burkholderia* sp. and a group similar to the genus *Herbaspirillum* could be detected in samples of both crops. However, *Azospirillum brasilense* and another two groups of *Herbaspirillum*-like bacteria were detected only in banana plants. Two isolates of the latter group were identified as *Herbaspirillum seropedicae*, whereas the other isolates may represent a new *Herbaspirillum* species.

Introduction

Fruit crops including banana and pineapple are cultivated in large areas of tropical countries. Considering the overall production of banana (54 million tons) and pineapple (11 million tons) in 1995, Brazil was responsible for the second and third highest production, respectively, for these crops (FAO, 1996).

Fruit crops have been frequently cultivated in soil of low fertility and to obtain high yields, fertilizer amounts, mainly nitrogen, are applied at levels over 200 $kg.ha^{-1}.year^{-1}$ (Quaggio and Raij, 1996). However, high doses of these fertilizers, as well as pesticides, adversely affect the environment as has been observed in banana cultivated areas (Risède and Muntcel, 1997).

An alternative to the use of nitrogen fertilizer could be the exploitation of the biological nitrogen fixation potential in agricultural systems. Benefits from dinitrogen fixation were already demonstrated in sugar cane (Urquiaga et al., 1992), rice (App et al., 1984) and gramineous forage plants (Boddey and Victoria, 1986). In addition, various genera of diazotrophic bacteria, such as *Beijerinckia*, *Azotobacter* and *Derxia* spp. have been isolated from the rhizosphere of several gramineous plants (Döbereiner and Ruschel, 1958; Döbereiner, 1966; Döbereiner and Campelo, 1971). Besides *Azospirillum* spp. (Döbereiner, 1977; Patriquin and Döbereiner, 1978) and *Herbaspirillum* spp.

* FAX No: 852991833. E-mail: weber@cnpat.embrapa.br

(Baldani et al., 1986; Gillis et al., 1991; Pimentel et al., 1991; Baldani et al., 1992; Baldani et al., 1996), more recently other bacterial genera have been isolated from internal tissues of roots and shoots of many gramineous plants. These include *Burkholderia* sp. (Hartmann et al., 1995) and *Acetobacter diazotrophicus* (Cavalcante and Döbereiner, 1988; Döbereiner et al. 1993). The poor survival in soil and the ability to colonize internal tissues of plants have suggested that these bacteria could belong to a new class of endophytic diazotrophic bacteria as revised by Baldani et al. (1997).

Not much attention has been given to the occurrence of nitrogen fixing bacteria in fruit crops. Only two reports show the presence of diazotrophs such as *Azospirillum* spp. in the rhizosphere of various fruit crops, including banana (Subba Rao, 1983) and pineapple plants (Ghai and Thomas, 1989). In this paper we demonstrate the occurrence and colonization of different parts of banana and pineapple plants grown in Brazil by different nitrogen-fixing bacteria.

Materials and methods

Plant genotypes and tissue sampling

Plants of banana (*Musa* spp.) cultivars 'Butuhan', 'Prata Anã', 'Yangambi' and pineapple (*Ananas comosus* (L.) Merril) cultivars 'Alenquer', 'Pérola' and 'Perolera' were harvested from the 'Germplasm Collection of Tropical Fruit Crop' at EMBRAPA – National Center for Cassava and Fruit Crop Research, in Cruz das Almas, Bahia (Brazil). Multiple duplicate samples of roots, stems and leaves of these crops were collected in June 1994 and January 1995. Banana samples were also collected from the cultivars 'Prata', 'D'Água', 'Maçã' and 'Marmelo' at the Porangaba farm in Itaguaí, Rio de Janeiro State (Brazil), while banana cultivars 'Prata Anã', 'Prata Manteiga' and 'D'Água' and pineapple cultivars 'Pérola' and 'Smooth Cayenne' were collected at the PESAGRO farm in the Macaé region, Rio de Janeiro (Brazil), in February 1996.

Enumeration and isolation of diazotrophic bacteria from plant material

Samples of roots, stems, leaves and fruits were washed in sterilized water and 1g of fresh material was macerated and diluted up to 10^{-5} in test tubes containing 9 ml of 1/4 salt solution of malate NFb medium with pH adjusted to 7.0 (Döbereiner et al., 1995). Aliquots of 0.1 ml were inoculated into vials containing 5 ml of semi-specific semi-solid, N-free medium, according to Döbereiner et al. (1995). Five days after incubation at approximately 30 °C, those vials showing a veil-like pellicle near the surface of the media were considered positive and used to estimate the amount of diazotrophs present in the sample by the MPN count technique. The cultures from the positive vials were subjected to further purification steps by streaking them onto specific agar plate containing 20 to 30 mg.l^{-1} of yeast extract. Distinct colonies grown on these media were randomly picked up and transferred to a fresh semi-solid N-free medium for final purification.

Physiological characteristics of diazotrophic bacterial isolates

The nitrogen fixation ability of the isolates was tested according to Döbereiner (1989). First, the isolates were inoculated in liquid malate NFb medium (Döbereiner et al., 1995) containing 0.1% of ammonium chloride and incubated with agitation at 120 rpm for 2 days at 30 °C. Once growth was observed, a new aliquot was transferred to a fresh NFB liquid medium and grown at the same time and conditions above. This procedure was repeated 10 times. After that, aliquots of 10 μl were transferred to vials containing semi-solid N-free media to evaluate the formation of a characteristic veil-like pellicle near the surface of the semi-solid N-free media. In addition, cells from these cultures were examined under the light microscope (400×) to evaluate their shape and movement.

The ability of a representative number of isolates to use different organic substrates was determined in semi-solid (with 1.7 g.l^{-1} agar) and liquid medium (with 0.1 g.l^{-1} of ammonium chloride). Carbon sources (5 g.l^{-1}) were dissolved in phosphate buffer (3 m*M*) adjusted to pH 5.8, filtered through a 0.2 μm pore sized sterile filter (Millipore) and added to the media. Aliquots of 15 μl inoculate from cultured isolates and reference strains were grown overnight in liquid DYGS-medium described by Rodrigues Neto et al. (1986) and modified by the addition of 1 g.l^{-1} of malate and adjustment of pH to 6.5. Before the inoculation, the cell suspension of all isolates was adjusted with sterilized water to an optical density (600 nm) around 0.75±0.02.

Table 1. Phylogenetic oligonucleotide probes used

Name/target	Sequence	Specificity	T_{Hyb} in °C	Reference
AA 23S rRNA	5′-ACACCTCCATGGCACAC-3′	*Azospirillum amazonense*	50	Kirchhof and Hartmann, 1992
AB 23S rRNA	5′-GGGTCCCCAGCCGGGC-3′	*Azospirillum brasilense*	55	Kirchhof and Hartmann, 1992
AL 23S rRNA	5′-TATAAGGCGGGGCTA-3′	*Azospirillum lipoferum*	40	Kirchhof and Hartmann, 1992
AZO 23S rRNA	5′-GGGGCT(A/G)TTTCC(C/T)GG-3′	genus *Azospirillum*	48	Kirchhof and Hartmann, 1992
BC16 16S rRNA	5′-CCTCTGTTCCGACCA-3′	*Burkholderia cepacia* and other closely related taxons	43	Leff et al., 1995
HR 23S rRNA	5′-ATGCAAAAACCGACTA-3′	*Herbaspirillum rubrisubalbicans*	40	Baldani et al., 1996
HS 23S rRNA	5′-ATGCAAAAACCGGGAC-3′	*Herbaspirillum seropedicae*	43	Baldani et al., 1996
M130 23S rRNA	5′-GCATTTCACGCCGGT-3′	*Burkholderia* sp., isolates from *Oryza sativa*	43	Kirchhof, unpublished
PPe8 23S rRNA	5′-CCTCGCACCCTTT-3′	*Burkholderia* sp., isolates from *Saccharum* spp.	37	Hartmann et al., 1995
ALF1b 16S rRNA	5′-CGTTCG(C/T)TCTGAGCCAG-3′	α-subgroup of *Proteobacteria*	50	Manz et al., 1992
BET42a 23S rRNA	5′-GCCTTCCCACTTCGTTT-3′	β-subgroup of Proteobacteria	47	Manz et al., 1992
Eub338 16S rRNA	5′-GCTGCCTCCCGTAGGAGT-3′	Bacteria	53	Amann et al., 1995

Phylogenetic analysis clustering of bacterial strains with oligonucleotide probes

Fruit crop isolates of N_2-fixing bacteria grown at 30 °C in the modified liquid DYGS medium were harvested at the log phase to extract the bulk nucleic acids. The extracting procedure was performed according to Kirchhof et al. (1998). After checking efficiency of bulk nucleic acids extraction on a 1% agarose gel, aliquots of 10 μl were dried, suspended in the same volume of a denaturing solution (100 μl MOPS 0.1M buffer, 500 μl formamide and 162 μl formaldehyde 37%), incubated for 10 min at 65 °C, cooled on ice and blotted on a positively charged nylon membrane. This membrane was baked at 80 °C for one hr to immobilize the bulk nucleic acids. After that, it was submitted to hybridization experiments with rRNA directed phylogenetic oligonucleotide probes (Kirchhof and Hatmann, 1992). The sequences of the oligonucleotides and hybridization temperatures used are listed in Table 1.

Results and discussion

Enumeration of diazotrophic bacteria in plants of banana and pineapple

Veil-like pellicles characteristic of N_2-fixing bacteria could be observed in all semi-solid N-free media inoculated with root, stem, leaf and fruit tissue samples from eight banana cultivars and four pineapple varieties. Nitrogen-fixing bacteria were observed in different dilutions and media used and varied between 10^{-2} and 10^{-6} per gram of fresh material (Table 2). Diazotrophic bacteria with similar population size have been detected in many gramineous plants using the same grown conditions above (Baldani et al., 1992; Barraquio et al., 1997; Kirchhof et al., 1997b).

Root samples collected in Cruz das Almas (Bahia State) showed the presence of diazotrophic bacteria in higher numbers, particularly when the semi-solid NFb medium was used. The malate N-free (NFb) medium has been frequently used to count and isolate strains of *A. lipoferum* and *A. brasilense* from cereals and forage plants (Döbereiner et al., 1995). Diazotrophs related to the genus *Azospirillum* were also detected on roots of various fruit crops when malate N-free medium was used (Subba Rao, 1983; Ghai and Thomas, 1989). In our study, similar numbers of diazotrophic bacteria were detected in root or stem samples of banana and pineapple plants collected in Rio de Janeiro using either the NFb or JNFb medium. According to Baldani et al. (1992) the latter medium is more specific to the genus *Herbaspirillum*, although *Azospirillum* can also grow and be isolated using the semi-solid JNFb medium.

The number of diazotrophic bacteria was lower in the LGI medium as compared to the NFb medium.

Table 2. Occurrence of diazotrophic bacteria in banana and pineapple plants collected in different regions of Brazil

Genotype	Local	Parts of the plants			
		Diazotrophic bacteria obtained from dilutions			
		Root	Stem	Leaf	Fruit
NFb medium[a]					
Banana	CA[b]	10^{-4}–10^{-6}	10^{-2}–10^{-4}	10^{-2}–10^{-4}	10^{-2}
Banana	I	10^{-4}–10^{-6}	10^{-4}–10^{-6}	10^{-2}–10^{-4}	10^{-2}
Banana	M	10^{-4}	10^{-4}	10^{-2}–10^{-4}	10^{-2}
Pineapple	CA	10^{-4}–10^{-6}	10^{-2}–10^{-4}	10^{-2}–10^{-4}	10^{-2}
Pineapple	M	10^{-2}–10^{-4}	10^{-2}	10^{-2}	ND[c]
LGI medium					
Banana	CA	10^{-2}–10^{-4}	10^{-2}	10^{-2}	10^{-2}
Banana	I	10^{-2}–10^{-4}	10^{-2}	10^{-2}–10^{-4}	10^{-2}
Banana	M	10^{-2}	10^{-2}–10^{-4}	10^{-2}	ND
Pineapple	CA	10^{-4}	10^{-2}–10^{-4}	10^{-2}–10^{-4}	10^{-2}
Pineapple	M	10^{-2}–10^{-4}	10^{-2}	10^{-2}	10^{-2}
JNFb medium					
Banana	I	10^{-4}–10^{-6}	10^{-2}–10^{-4}	10^{-2} a 10^{-4}	10^{-2}
Banana	M	10^{-4}	10^{-4}	10^{-2} a 10^{-4}	10^{-2}
Pineapple	M	10^{-2}–10^{-4}	10^{-2}	10^{-2}	10^{-2}
JMV medium					
Banana	I	10^{-2}–10^{-6}	10^{-2}–10^{-4}	10^{-2} a 10^{-4}	10^{-2}
Banana	M	10^{-2}	10^{-2}–10^{-4}	10^{-2}	ND
Pineapple	M	10^{-4}	10^{-2}–10^{-4}	10^{-2}	10^{-2}

[a] Carbon source of NFb and JNFb media are malate; LGI medium is sucrose; and JMV medium is mannitol.
[b] Washed samples from plants harvested in Cruz das Almas (CA), Itaguaí (I) and Macaé (M) regions.
[c] Growth not detected (ND) after 5 days of incubation at 30 °C.

This suggests a lower population of *A. amazonense* in banana and pineapple plants. The main carbon source for *A. amazonense* N_2-dependent growth is sucrose, which is a carbon source also present in these fruit crops (data not shown). However, malate can also be used by this species when the pH of the medium does not become alkaline (Döbereiner and Pedrosa 1992). We also detected diazotrophic bacteria in an acid mannitol medium (JMV) (Baldani,1996) from most of the banana and pineapple plants collected in the Itaguaí and Macaé region. This suggested the presence of diazotrophic bacteria of the genus *Burkholderia* in these fruit crops.

Physiological characteristics of diazotrophic bacteria

Cultures of diazotrophic bacteria isolated from banana and pineapple samples (Table 3) were purified and grouped according to their morphological and physiological characteristics (Tables 4 and 5). All isolates were able to keep their ability to form veil-like pellicles in the semi-solid media after 10 successive transfers to rich liquid medium, indicating that the nitrogen-fixing characteristic is inherited by these bacteria and does not represent only a transient characteristic of the isolates.

The ability of the bacterial groups to use different carbon sources was evaluated in semi-solid and also in liquid medium. Isolates from the group I showed large white and flat colonies with elevated margins typical of *A. amazonense* when grown on solid potato agar medium (Döbereiner et al., 1995). However, they also use D-fructose when evaluated in semi-solid (Table 4) or liquid (Table 5) medium. This substrate is neither used by the type strain *A. amazonense* $Y6^T$, nor by other strains of this species when grown under N_2-dependent conditions (Magalhães et al., 1983). Nevertheless, auxanographic tests have shown their

Table 3. Grouping of diazotrophic bacteria isolated from banana and pineapple plants based on their morphological and physiological characteristics

Genotype	Sample	Medium	Dilution	Local	No. of isolates
Azospirillum amazonense (group I)					
Banana	Root	LGI	10^{-2} to 10^{-4}	I[a]	3
Pineapple	Root	LGI	10^{-4}	CA, M	2
Azospirillum brasilense (group II)					
Banana	Root	NFb	10^{-6}	CA	3
Azospirillum lipoferum (group III)					
Banana	Root	NFb, JNFb	10^{-4} to 10^{-6}	CA, I	3
	Leaf	NFb	10^{-2} to 10^{-4}	I	1
Pineapple	Root	NFb	10^{-4} to 10^{-6}	CA	2
	Leaf	NFb	10^{-4}	CA	1
Herbaspirillum-like bacteria (group IV)					
Banana	Root	NFb, JNFb	10^{-4} to 10^{-6}	I	2
	Stem	NFb, JNFb	10^{-2} to 10^{-6}	CA, I, M	7
	Leaf	NFb, JNFb	10^{-4}	CA, I	2
	Fruit	JNFb	10^{-2}	I	1
Pineapple	Stem	NFb	10^{-4}	CA	1
	Leaf	NFb	10^{-6}	CA	1
Herbaspirillum-like bacteria (group V)					
Banana	Root	NFb	10^{-4} to 10^{-6}	CA, I	3
	Stem	NFb, JNFb	10^{-4}	CA, I	3
	Leaf	NFb, JNFb	10^{-2} to 10^{-4}	CA, I, M	4
	Fruit	JNFb	10^{-4}	I	1
Herbaspirillum-like bacteria (group VI)					
Banana	Root	NFb	10^{-4}	I	1
	Stem	NFb, JNFb	10^{-4}	CA, I, M	5
	Leaf	NFb, JNFb	10^{-2}	CA, I	6
	Fruit	NFb	10^{-2}	I	1
Burkholderia-like bacteria (Group VII)					
Banana	Root	LGI, JMV	10^{-2} to 10^{-4}	CA, M	4
	Stem	JMV	10^{-2}	I, M	3
	Leaf	JMV	10^{-4}	I	1
	Fruit	LGI	10^{-2}	CA	1
Pineapple	Root	LGI, JMV	10^{-4}	CA, M	5
	Stem	LGI, JMV	10^{-2} to 10^{-4}	CA, M	5
	Leaf	JMV	10^{-4}	M	1
	Fruit	LGI	10^{-2}	M	1

[a] Samples from plants harvested in Cruz das Almas (CA), Itaguaí (I) and Macaé (M) regions.

ability to catabolise D-fructose (Gillis and Reinhold Hurek, 1994).

Isolates from group II and III showed morphological and physiological characteristics resembling *A. brasilense* and *A. lipoferum* when cultivated in semi-solid N-free medium. Isolates from group II seemed to be more restricted in their ability to use carbon sources as compared to the species *A. lipoferum* and *A.*

Table 4. Carbon sources uses for N_2-dependent growth of different isolates obtained from banana and pineapple plants

Carbon source	Groups and reference strains[b]												
	I	Y6^T	II	Sp7^T	III	Br17^T	IV	Z67^T	V	M4^T	VI	VII	M130[c]
D-Raffinose[a]	–	–	–	–	–	–	–	–	–	–	–	V[e]	+++
	(3)[d]		(3)		(9)		(11)		(11)		(11)	(13)	
D-Maltose	+++	+++	–	–	–	–	–	–	–	–	–	–	–
	(7)		(3)		(9)		(5)		(5)		(8)	(6)	
D-Sucrose	+++	+++	–	–	–	–	–	–	–	–	–	+++	++
	(7)		(3)		(9)		(16)		(12)		(14)	(18)	
D-Glucose	+++	+++	–	–	+++	+++	++	+++	+++	+++	–	+++	+++
	(5)		(3)		(9)		(11)		(9)		(11)	(12)	
D-Fructose	+++	–	+++	+++	+++	+++	+	+	+	+	–	+++	+++
	(5)		(3)		(9)		(10)		(11)		(9)	(11)	
D-Galactose	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	–	+++	+++
	(3)		(3)		(9)		(6)		(6)		(5)	(10)	
L-Arabinose	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	–	+++	+++
	(3)		(3)		(9)		(11)		(4)		(9)	(13)	
D-Mannitol	–	–	–	–	+++	+++	++	+++	++	+++	+	++	++
	(5)		(3)		(9)		(17)		(12)		(8)	(15)	
Glycerol	–	–	+	+	+++	+++	++	+++	++	++	++	++	++
	(3)		(3)		(5)		(4)		(5)		(6)	(7)	
D-Sorbitol	–	–	–	–	+++	+++	++	+++	++	+++	++	+++	++
	(3)		(3)		(6)		(10)		(11)		(8)	(13)	
Meso-ery-	–	–	–	–	–	–	–	–	+++	+++	–	V	–
thritol+NH_4	(5)		(3)		(5)		(17)		(12)		(13)	(16)	
N-acetyl-	–	–	–	–	+++	+++	+++	+++	–	–	–	+++	+++
glucosamine	(4)		(3)		(9)		(17)		(12)		(13)	(16)	
Citrate	–	–	–	–	+++	+++	+++	+++	+++	+++	++	+++	+++
	(2)		(3)		(5)		(5)		(6)		(7)	(4)	
α-ceto-	+++	+++	++	++	+++	+++	++	+++	+++	+++	++	+	–
glutarate	(3)		(3)		(5)		(4)		(6)		(7)	(6)	
Oxalate	–	–	+++	+++	+	+	–	–	–	–	–	+	–
	(4)		(3)		(4)		(8)		(8)		(8)	(6)	
Na D-	–	–	+	+	+	+	+++	+++	+++	+++	++	++	++
Gluconate	(4)		(3)		(4)		(4)		(4)		(8)	(6)	
L-Tartrate	–	–	–	–	–	–	++	–	==	–	–	–	+
	(4)		(3)		(7)		(12)		(11)		(13)	(13)	

[a] Nitrogen free medium, except for meso-erythritol and acetil-glucosamine.
[b] All isolates were able to grow in succinate, fumarate and malate.
[c] Reference strains: *Azospirillum amazonense* (Y6^T), *Azospirillum brasilense* (Sp7^T), *Azospirillum lipoferum* (Br17^T), *Herbaspirillum seropedicae* (Z67^T), *Herbaspirillum rubrisubalbicans* (M4^T) and *Burkholderia* sp. (M130).
[d] Number of isolates (in parenthesis) with good (+++), medium (++), poor (+), no growth (–) after 5 days of incubation at 30 °C.
[e] Variable (v) growth among isolates.

amazonense. Bacteria from group II were only detected in banana plants (Table 3). Three bacterial groups (IV, V and VI) are thin rods similar to those observed in strains of the genus *Herbaspirillum*. Isolates from group IV (Table 4 and 5) showed the ability to use the same carbon sources as *H. seropedicae*. The only exception was their ability to grow with L-tartrate, a substrate not so far known to be used by the *Herbaspirillum* species (Baldani et al., 1996). Kirchhof et al. (1997a) also observed different physiological characteristics in *Herbaspirillum*-like bacteria isolated from *Miscanthus* when tartrate was evaluated under N_2-dependent condition.

Isolates from group V, detected only in banana plants, also grew with L-tartrate as carbon source in semi-solid media (Table 4) as well as liquid

Table 5. Carbon sources use by the different groups of isolates obtained from banana and pineapple plants and reference strains in liquid media

Carbon source	Groups and reference strains[d]												
	I[a]	Y6^T	II	Sp7 ^{rmT}	III	Br17^T	IV	Z67^T	V	M4^T	VI	VII	M130[b]
D-Raffinose	–	–	–	–	–	–	–	–	–	–	–	+[c]	UN
	(5,7)	(5,7)	(5,8)	(5,8)	(5,8)	(5,8)	(5,7)	(5,6)	(5,7)	(5,8)	(5,7)	(3,1)	
D-Maltose	+	+	–	–	–	–	–	–	–	–	–	–	–
	(3,8)	(3,7)	(5,7)	(5,7)	(5,7)	(5,6)	(5,6)	(5,7)	(5,7)	(5,6)	(5,7)	(5,7)	(5,6)
D-Sucrose	+	+	–	–	–	–	–	–	–	–	–	+	+
	(3,8)	(3,7)	(5,7)	(5,7)	(5,7)	(5,7)	(5,7)	(5,7)	(5,7)	(5,6)	(5,7)	(3,0)	(5,4)
D-Glucose	+	+	–	–	+	+	+	+	+	+	–	+	+
	(3,6)	(3,6)	(5,7)	(4,8)	(3,7)	(3,7)	(3,4)	(3,5)	(3,5)	(3,3)	(5,7)	(3,0)	(3,9)
D-Fructose	+	–	+	+	+	+	+	+	+	+	+	+	+
	(3,5)	(5,7)	(3,9)	(4,0)	(3,8)	(3,6)	(3,7)	(3,6)	(3,7)	(3,8)	(3,8)	(4,0)	(3,1)
D-Galactose	+	+	+	+	+	+	+	+	+	+	–	+	+
	(3,9)	(3,5)	(5,6)	(4,9)	(4,4)	(4,6)	(3,6)	(3,7)	(3,7)	(3,6)	(5,6)	(3,1)	(3,7)
L-Arabinose	+	+	+	+	+	+	+	+	+	+	–	+	+
	(3,9)	(5,1)	(5,4)	(5,5)	(5,1)	(5,6)	(5,2)	(4,9)	(4,6)	(5,2)	(5,6)	(3,2)	(3,1)
D-Mannitol	–	–	–	–	+	+	+	+	+	+	+	+	+
	(5,7)	(5,7)	(5,7)	(UN)	(4,0)	(3,8)	(3,4)	(3,5)	(3,6)	(3,5)	(4,1)	(3,1)	(3,0)
Glycerol	–	–	–	+	+	+	+	+	+	+	+	+	+
	(5,2)	(5,6)	(5,4)	(5,1)	(4,1)	(3,9)	(3,3)	(3,4)	(3,3)	(3,2)	(3,5)	(3,5)	(3,1)
D-Sorbitol	–	–	–	–	+	+	+	+	+	+	+	+	+
	(5,8)	(5,8)	(5,6)	(5,7)	(4,2)	(4,1)	(3,8)	(3,7)	(3,8)	(3,5)	(4,6)	(3,0)	(3,2)
Meso-ery-thritol+NH_4	–	–	–	–	–	–	–	–	+	+	–	+	–
	(5,8)	(5,9)	(5,8)	(5,8)	(5,8)	(5,8)	(5,8)	(5,8)	(3,4)	(5,3)	(5,8)	(3,0)	(5,8)
N-acetyl-glucosamine	–	–	–	–	+	+	+	+	–	–	–	+	+
	(5,8)	(5,7)	(5,6)	(5,8)	(5,7)	(5,5)	(6,4)	(6,1)	(5,7)	(5,7)	(5,7)	(6,3)	(6,4)
Citrate	–	–	–	–	+	+	+	+	+	+	+	+	+
	(5,9)	(5,9)	(5,8)	(5,9)	(7,9)	(8,2)	(8,8)	(8,8)	(9,0)	(8,5)	(8,7)	(8,2)	(UN)
α-ceto-glutarate	+	+	+	+	+	+	+	+	+	+	+	+	+
	(UN)	(UN)	(6,9)	(UN)	(UN)	(UN)	(8,6)	(8,2)	(8,2)	(8,8)	(UN)	(8,3)	(6,8)
Oxalate	–	–	+	+	+	–	–	–	–	–	–	+++	–
	(6,1)	(6,3)	(8,0)	(8,1)	(8,5)	(9,1)	(5,8)	(5,8)	(5,9)	(6,1)	6,0)	(7,5)	(6,2)
Na D-Gluconate	–	–	+	+	+	+	+	+	+	+	+	+	+
	(5,8)	(5,8)	(6,1)	(6,0)	(6,1)	(6,0)	(7,1)	(7,2)	(7,2)	(6,9)	(7,3)	(7,2)	(7,3)
L-Tartrate	–	–	–	–	–	–	+	–	+	–	–	–	+
	(5,9)	(5,9)	(5,8)	(5,8)	(5,8)	(5,8)	(8,1)	(6,1)	(8,4)	(5,9)	(6,0)	(6,0)	(7,1)

[a] Isolates from group I (AB44), II (BA5 and BA6), III (AB3 and BA52), IV (BA10 and BA11), V (BA13 e BA14), VI (BA22 e BA23), and VII (AB 31 and AB33).
[b] Reference strains: *Azospirillum amazonense* (Y6 ^{rmT}), *Azospirillum brasilense* (Sp7^T), *Azospirillum lipoferum* (Br17^T), *Herbaspirillum seropedicae* (Z67^T), *Herbaspirillum rubrisubalbicans* (M4^T) and *Burkholderia* sp. (M130).
[c] Absorbance at 600 nm >0.05 (+) and <0.05 (–) with the final pH in the medium, and growth undetermined (UN) after 48 h incubation at 30 °C.
[d] All isolates grow well in substrate with succinate, fumarate and malate and produced alkaline media (pH >8.0).

(Table 5) medium. On the other hand, bacteria from group VI, isolated from banana plants, differed in their ability to grow on carbon sources from the already known *Herbaspirillum* species. This group of bacteria was not able to grow with D-glucose, D-galactose, L-arabinose, meso-erythritol+NH_4 or N-acetyl-glucosamine, which are carbon sources normally used by strains of the genus *Herbaspirillum*. They also differed from diazotrophic bacteria from the genera *Pseudomonas* (Watanabe et al., 1987), *Acetobacter* (Gillis et al., 1989), *Azoarcus* (Reynold Hurek et al., 1993), *Burkholderia* (Gillis et al., 1995) and

Table 6. Hybridization of nucleic acids of diazotrophic bacteria isolated from banana (B) and pineapple (P) plants and reference strains with phylogenetic oligonucleotide probes

Strain	Origin	Target probe												
		ALF1b	BET42a	AZO	AA	AB	AL	HS	HR	beta20	BC16	Ppe8	M130	Eu338
		16S rRNA	23S rRNA	23S rRNA	23S rRNA	23S rRNA	23S rRNA	23S rRNA	23S rRNA	23S rRNA	16S rRNA	23S rRNA	23S rRNA	16S rRNA
		Reactions[b,c]												
Azospirillum amazonense (group I)														
AB44	P. Alenquer-root	+[a]	–	+	+	–	–	–	–	–	–	–	UN	+
Y6^T	*P. purpureum*-root	+	–	+	+	–	–	–	–	–	–	–	–	+
Azospirillum brasilense (group II)														
BA5	B. Yangambi-root	+	–	+	–	+	–	–	–	–	–	–	UN	+
Sp7^T	*B. decumbens*-root	+	–	+	–	+	–	–	–	–	–	–	–	+
Azospirillum lipoferum (group III)														
AB2	P. Alenquer-leaf	+	–	+	–	–	+	–	–	–	–	–	UN	+
Br17	*Zea mays*-root	+	–	+	–	–	+	–	–	–	–	–	–	+
Herbaspirillum seropedicae (Group IV-1)														
BA153	B. Marmelo-fruit	–	+	–	UN	UN	UN	+	–	–	–	–	–	+
Z67^T	*Oryza sativa*-root	–	+	–	–	–	–	+	–	–	–	–	–	+
Herbaspirillum sp. (Group IV-2)														
BA135	B. Maçã-root	–	+	–	UN	UN	UN	–	–	+	–	–	–	+
β20	*Miscanthus* sp. stem	–	+	–	–	–	–	–	–	+	–	–	–	+
Burkholderia cepacia (Group VII-1)														
AB33	P. Pérola-root	–	+	–	–	–	–	–	–	–	+	–	UN	+
AB119	P. Pérola-leaf	–	+	–	UN	UN	UN	–	–	–	+	–	–	+
Burkholderia sp. (Group VII-2)														
AB98	P. Pérola-fruit	–	+	–	–	–	–	–	–	–	+	+	–	+
Ppe8	*Saccharum* sp. stem	–	+	–	–	–	–	–	–	–	+	+	–	+
Burkholderia sp. (Group VII-3)														
BA123	B. P. Manteiga-root	–	+	–	UN	UN	UN	–	–	–	+	–	+	+
M130	*Oryza sativa*-root	–	+	–	–	–	–	–	–	–	+	–	+	+

[a] Positive (+), negative (–) reactions and analysis undetermined (UN) of bulk bacterial nucleic acids.
[b] Similar reactions of NA from bacteria in group II (BA5, BA6 and BA60); group III (AB2, AB3, AB53, BA4, BA52, BA102 and BA148); group IV-1) (BA153 and BA155); group VII-1 (AB31, AB33, AB147, BA67 and BA69; AB119, AB120, AB146, BA112 and BA151); group VII-2 (AB98, AB147 and AB48); group VII-3 (BA123, AB116, AB122, BA124 and BA126).
[c] No reactions of NA from *Herbaspirillum*-like bacteria: AB7, AB9, BA10, BA11, BA12, BA13, BA14, BA15, BA16, BA22, BA23, BA24, BA25, BA85, BA88, BA103, BA104, BA128 with oligonucleotide probes directed to *Azospirillum*, *Herbaspirillum* and *Burkholderia* species and *Acetobacter diazotropicus*.

Sphingomonas (Kämpfer et al., 1997) in relation to their carbon source utilization pattern.

Isolates from group VII showed cell shape similar to bacteria within the genus *Burkholderia* and a high diversity in their ability to use the carbon substrates (Tables 4 and 5). However, only few isolates revealed the ability to use D-raffinose and meso-erythritol+NH_4 as sole carbon source. According to Gillis et al. (1995), *B. cepacia, B. caryophilli, B. glumae* and *B. vietnamiensis* can use D-raffinose as the sole carbon source. The species *B. vietnamiensis,* is a nitrogen-fixing bacterium isolated from rhizosphere soil of rice plants grown in Vietnam (Gillis et al., 1995). Another diazotrophic bacteria representative of the genus *Burkholderia* have recently been isolated from rice plants and sugar cane plants and provisorily named *Burkholderia brasilensis*. This new proposed specie is able to use mannitol and D-raffinose but could not growth with meso-erythritol+NH_4 and is able to grow at pH values in the range of 4.0 to 6.4 (Oliveira, 1992; Baldani, 1996; Baldani et al., 1997).

Genotyping diazotrophic bacteria strains with oligonucleotide probes

The occurrence of three species of the *Azospirillum* genus in association with banana and pineapple plants was demonstrated by the hybridization experiments of genera and species-specific oligonucleotide probes against the bulk nucleic acid extracted from the isolates. (Table 6). These results are in agreement with the physiological characteristics observed before in groups I to III (Tables 4 and 5). Bulk nucleic acid extracts from these three groups of diazotrophs showed positive signals against probes directed to 23S rRNA of the *Azospirillum* genus and 16S rRNA of the alpha-subclass. According to Manz et al. (1992) and Amann et al. (1995), this confirms the affiliation of these bacterial groups within the genus *Azospirillum* and the alpha-subclass of *Proteobacteria*.

Isolates from groups IV to VI (*Herbaspirillum*-like bacteria) could be included in the beta-subclass of *Proteobacteria* (Table 6). A positive signal was observed with the probe directed to the 23S rRNA designed for identification of this subclass. However, only two strains isolated from banana plants were identified as *H. seropedicae*. On the other hand, one isolate recovered from banana plants (group IV) was affiliated to the beta-20 bacterial group. This *Herbaspirillum*-like bacterium was originally isolated from a Miscanthus plant, an energy crop grown in Germany (Kirchhof et al., 1997a). Species-specific oligonucleotide probes, available in our laboratory and designed for identification of known diazotrophic bacteria, showed no signal when hybridized against the bulk nucleic acids of all other isolates from the *Herbaspirillum*-like bacterial groups (group IV to VI). The oligonucleotide probe technique confirmed at molecular level the association of *H. seropedicae* with banana plants.

In contrast, extracts of bulk nucleic acids from group VII isolates reacted with the probe directed to 16S rRNA of *B. cepacia* and the probe designed specifically for the beta-subclass of *Proteobacteria* (Table 6). However, five isolates from the same group were related to the cluster of strain M130, a representative of *B. brasilensis* (Baldani, 1996; Baldani et al., 1997). Three other isolates from the same physiological group were related to the cluster of strain Ppe8, a strain isolated from sugar cane plants and also belonging to the genus *Burkholderia* and which may constitute another new species (Hartmann et al., 1995). These results suggest that isolates from physiological bacterial group VII, are related *to B. cepacia* but it may also represents more than one species of diazotrophs and additional polyphasic studies are needed to identify their correct position within the beta-subclass of *Proteobacteria*.

Although these bacteria were isolated from non-sterilized tissues of both plants, they were frequently detected at innermost tissues harvested from the pineapple fruit and banana stems. Many of the diazotrophs identified here are naturally found associated with gramineous plants. Because of their ability to colonize the interior of the plant tissues and to show lower survival in soil some of them have been included in the group of endophytic bacteria (Döbereiner, 1992). The possibility that many of these isolates obtained from banana and pineapple plants belong to the group of diazotrophic endophytes should not be discarded since preliminary microscope studies have shown the tissue interior colonization of micropropagated plants inoculated by these strains (Weber, in preparation).

These results constitute the first report of the occurrence of diazotrophic bacteria on the surface and in roots, stem, leaves and fruits of banana and pineapple plants and show a large diversity of nitrogen fixing bacteria colonizing these fruit crops. However, further studies need to be carried out to confirm their phylogenies and their biological nitrogen fixation contribution to the plants.

References

Amann R I, Ludwig W and Schleifer K H 1995 Phylogenetic identification and *in situ* detection of individual microbial cells without cultivation. Microbiol. Rev. 59, 143–169.

App A, Santiago T, Daez C, Menguito G, Ventura W, Tirol A, Po J, Watanabe I, De Datta S K and Roger P 1984 Estimation of the nitrogen balance for irrigated rice and the contribution of phototrophic nitrogen fixation. Field Crop Res. 9, 17–27.

Baldani J I, Baldani V L D, Seldin L and Döbereiner J 1986 Characterization *of Herbaspirillum seropedicae* gen. nov., a root-associated nitrogen-fixing bacterium. Int. J. Sys. Bacteriol. 36, 86–93.

Baldani J I, Caruso L, Baldani V L D, Goi S R and Döbereiner J 1997 Recent advances in BNF with non-legume plants. Soil Biol. Biochem. 29, 911–922.

Baldani J I, Pot T B, Kirchhof G, Falsen E, Baldani V L D, Olivares F L, Hoste B, Kersters K, Hartmann A, Gillis M and Döbereiner J 1996 Emended description of *Herbaspirillum*; a mild plant pathogen, as *Herbaspirillum rubrisubalbicans* comb. nov.; and classification of a group of clinical isolates (EF group 1) as *Herbaspirillum species 3*. Int. J. Syst. Bacteriol. 46, 802–810.

Baldani V D L, Oliveira E, Balota E, Baldani J I, Kirchhof G and Döbereiner J 1997 *Burkhoderia brasilensis* sp. nov., uma espécie de bactéria diazotrófica endofítica. An. Acad. brasi. Ci. 69, 116.

Baldani V L D 1996. Efeito da inoculação de *Herbaspirillum* spp. no processo de colonização e infecção de plantas de arroz e, ocorrência e caracterização parcial de uma nova bactéria diazotrófica. pp 238. UFRRJ, Seropédica. PhD thesis.

Baldani V L D, Baldani J I, Oliveira F and Döbereiner J 1992 Identification and ecology of *Herbaspirillum seropedicae* and the closely related *Pseudomonas rubrisubalbicans*. Symbiosis 13, 65–73.

Barraquio W L, Revilla L and Ladha J K 1997 Isolation of endophitic diazotrophic bacteria from wetland rice. Plant Soil 194, 15–24.

Boddey R M and Victoria R L 1986 Estimation of biological nitrogen fixation associated with *Brachiaria* and *Paspalum* grasses using ^{15}N labeled organic matter and fertilizer. Plant Soil 90, 265–292.

Cavalcante V A and Döbereiner J 1988 A new acid-tolerant nitrogen-fixing bacterium associated with sugarcane. Plant Soil 108, 23–31.

Döbereiner J 1966 *Azotobacter paspali* sp. n., uma bactéria fixadora de nitrogênio na rizosfera de *Paspalum*. Pesquisa Agro. brasi. 1, 357–365.

Döbereiner J 1977 Fixação de nitrogênio em gramíneas. Rev. brasi. Ci. Solo 1, 1–54.

Döbereiner J 1989 Isolation and identification of root associated diazotrophs. Plant Soil 110, 207–212.

Döbereiner J 1982 History and new perspectives of diazotrophs in association with non-leguminous plants. Symbiosis 13, 1–13.

Döbereiner J and Campelo A B 1971 Non-symbiotic nitrogen fixing bacteria in tropical soils. Plant Soil v. special, 457–470.

Döbereiner J and Pedrosa F O 1992 Nitrogen-fixing bacteria in nonleguminous crop plants. 155 pp. Springer-Verlag, Madison.

Döbereiner J and Ruschel A P 1958 Uma nova espécie de *Beijerinckia*. Rev. Biol. 1, 261–272.

Döbereiner J, Baldani V L D and Baldani J I 1995 Como isolar e identificar bactérias diazotróficas de plantas não-leguminosas. 60 pp. Embrapa-SPI, Brasília.

Döbereiner J, Reis V M, Paula M A and Olivares F 1993 Endophytic diazotrophs in sugar cane, cereals and tuber plants. *In* New Horizons in Nitrogen Fixation. Eds Palacios R, Mora J and Newton W E. pp 671-676. Kluwer Academic Publ., Dordrecht.

FAO 1996 Production Yearbook 49, 164–167.

Ghai S K and Thomas G V 1989 Occurrence of *Azospirillum* spp. in coconut-based farming systems. Plant Soil 114, 235–241.

Gillis M, Döbereiner J, Pot B, Good M, Falsen E, Hoste B, Reinhold B and Kersters K 1991 Taxonomic relationships between (*Pseudomonas) rubrisubalbicans*, some clinical isolates (EF group I), *Herbaspirillum seropedicae* and (*Aquaspirillum*) *autotrophicum*. Dev. Plant Soil Sci. 48, 293–294.

Gillis M, Kersters K, Hoste B, Janssens D, Kroppenstedt R M, Stephan M P, Teixeira K R S, Döbereiner J and Ley J 1989 *Acetobacter diazotrophicus* sp. nov., a nitrogen fixing acetic acid bacterium associated with sugarcane. Int. J. Syst. Bacteriol. 39, 361–364.

Gillis M and Reinhold Hurek B 1994 Taxonomy of *Azospirillum. In Azospirillum*/plant associations. Ed. Y Okon. pp 1–14. CRC Press, Boca Raton.

Gillis M, Van T V, Bardin R, Goor M, Hebbar P, Willems A, Segers P, Kersters K, Heulin T and Fernandez M P 1995 Polyphasic taxonomy in genus *Burkholderia* leading to an emended description of the genus and proposition of *Burkholderia vietnamiensis* sp: nov. for N2-fixing isolates from rice in Vietnam. Int. J. Syst. Bacteriol. 45, 274–289.

Hartmann A, Baldani J I, Kirchhof G, Assmus B Hutzler P, Springer N, Ludwig G W, Baldani V L and Döbereiner J 1995 Taxonomic and ecological studies of diazotrophic rhizosphere bacteria phylogenetic probes. *In Azospirillum* VI and Related Microorganisms: Genetics, Physiology and Ecology. Eds Fendrik I, Del Gallo M, Vanderleyden J, Zamaroczy M. pp 416–427. Springer-Verlag, New York.

Kämpfer P, Denner E B M, Meyer S, Moore E R B and Busse H 1997 Classification of '*Pseudomonas azotocolligans*' Anderson 1995, 132, in the genus *Sphingomonas* as *Sphingomonas trueperi* sp. nov. Int. J. Syst. Bacteriol. 47, 577–583.

Kirchhof G and Hartmann A 1992 Development of gene-probes for *Azospirillum* based on 23S-rRNA sequences. Symbiosis 13, 27–35.

Kirchhof G, Baldani J I, Reis V M and Hartmann A 1998. Molecular assay to identify *Acetobacter diazotrophicus* and to detect its occurrence in plant tissues. Can. J. Microbiol. 44, 12–19.

Kirchhof G, Reis V M, Baldani J I, Eckert B, Döbereiner J and Hartmann A 1997a Occurrence, physiological and molecular analysis of endophytic diazotrophic bacteria in gramineous energy plants. Plant Soil 194, 45–55.

Kirchhof G, Schloter M, Assmus B and Hartmann A 1997b Molecular microbial ecology approaches applied to diazotrophs associated with non-legumes. Soil Biol. Biochem. 29, 853–862.

Leff L G, Kernan R M, McArthur J V and Shimkets L J 1995 Identification of aquatic *Burkholderia* (*Pseudomonas*) *cepacia* by hybridization with species-specific rRNA gene probes. Appl. Env. Microbiol. 61, 1634–1636.

Magalhães F M, Baldani J I, Souto S M, Kuykendall J R and Döbereiner J 1983 A new acid-tolerant *Azospirillum* species. An. Acad. brasi. Ci. 55, 417–430.

Manz W, Amann R, Ludwig W, Wagner M and Schleifer K 1992 Phylogenetic oligodeoxynucleotide probes for the major subclasses of Proteobacteria: Problems and solutions. System. Appl. Microbiol. 15, 593–600.

Oliveira E 1992. Estudo da associação entre bactérias diazotroficas e arroz (*Oryza sativa*, L.) inundado. 96 pp. UFRRJ, Rio de Janeiro. MSc Thesis.

Patriquin D G and Döbereiner J 1978 Light microscopy observation of tetrazolium reducing bacteria in endorhizosphere of maize and other grasses in Brazil. Can. J. Microbiol. 24, 734–742.

Pimentel J P, Olivares F, Pitard R M, Urquiaga S, Akiba F and Döbereiner J 1991 Dinitrogen fixation and infection of grass leaves by *Pseudomonas rubrisubalbicans* and *Herbaspirillum seropedicae*. Plant Soil 134, 61–65.

Quaggio J A and Raij B 1996 Frutíferas *In* Recomendações de Adubação e Calagem para o Estado de São Paulo. Eds. Raij B van, Canterella H, Quaggio J A, Furlani A M C. pp 119–153. IAC, Campinas.

Reinhold Hurek B, Hurek T, Gillis M, Hoste B, Vancanneyt M, Kersters K and Ley J 1993 *Azoarcus* gen. nov., Nitrogen-fixing Proteobacteria associated with roots of kallar grass (*Leptochloa fusca* (L.) Kunth), and description of two species, *Azoarcus indigens* sp. nov. and *Azoarcus communis* sp. nov. Int. J. Syst. Bacteriol. 43, 574–584.

Risède M N and Muntcel H T 1997 Systèms monoculturaux bananiers et protection de l'environnement: état des lieux et perspectives. Fruits 52, 225–232.

Rodrigues Neto J, Malavolta Jr V A and Victor 1986 O Meio simples para isolamento e cultivo de *Xanthomonas campestris* pv. citri Tipo B. Sum. Phytol. 2, 16.

Subba Rao N S 1983 Nitrogen-fixing bacteria associated with plantation and orchard plants. Can. J. Microbiol. 29, 863–866.

Urquiaga S, Cruz K H S and Boddey R M 1992 Contribution of nitrogen fixation to sugar cane: nitrogen-15 and nitrogen balance estimates. Soil Sci. Soc. Am. J. 56, 105–114.

Watanabe I, So R and Ladha J K 1987 A new nitrogen-fixing species of pseudomonad: *Pseudomonas diazotrophicus* sp. nov. isolated from the root of wetland rice. Can. J. Microbiol. 33, 670–678.

Section editor: F R Minchin